Cyanobacteria attack rocks (CATS): Control and preventive strategies to avoid damage caused by cyanobacteria and associated microorganisms in Roman hypogean monuments

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ABSTRACT: The CATS project focuses on the control, prevention and monitoring of cyanobacteria-dominated biofilms that cause damage to rock surfaces in Roman hypogea. It develops and integrates physical and biotechnological methods intended to limit the growth of microorganisms on valuable archaeological surfaces, and applies analytical methods to monitor the presence and the extent of the microbial damage. As in other hypogea, the development of biofilms is favoured by the limited air circulation, the even temperature throughout the year, and the high level of humidity. Biofilms composed of sciaphilous chroococcal and filamentous cyanobacteria associated with other microorganisms develop thanks to the light gradients that occur in the proximity of entrances and artificial lamps. Terrestrial cyanobacteria and associated microorganisms are the first colonisers of exposed lithic faces and their extensive development is supported by the mineral composition of the substrata and facilitated by the porous nature of the, mostly calcareous, surfaces.

1 INTRODUCTION

Although atmospheric pollution is generally recognised as a significant factor in the deterioration of cultural properties and, consequently, has been thoroughly investigated in recent years, stone biodeterioration deserves more attention that it has so far received. In fact, a great variety of microorganisms colonising stones in different environments have been recorded (Ortega-Calvo et al. 1993, Hirsch et al. 1995, Tomaselli et al. 2000, De Leo & Urzì 2003). Bacteria and cyanobacteria can be found both on and beneath surfaces in indoor or outdoor environments. These microorganisms develop not as single colonies but they are components of complex systems called microbial mats or biofilms. However, the interactions between different microorganisms within the biofilm, the use of organic pollutants as nutrients, the different inputs of organic matter, and a host of other factors have barely been investigated (Saiz-Jimenez 1995b, 1997, Ortega-Calvo et al. 1995, Ascaso et al. 1998, Warscheid & Braams 2000, Dornieden et al. 2000a). Indeed, in general terms, the role of bacteria (including cyanobacteria) in biodeterioration processes is far from being understood. Accordingly, archaeological hypogea, and the works of art that they contain, need to be protected against the biodeteriorative action of microorganisms developing on rock surfaces (Agarossi et al. 1985, Albertano 1991, 1998, Ariño et al. 1997). The wide distribution of these archaeological remains in Southern Europe, and their cultural, artistic and religious importance, emphasises the potential social impact that biological damage can wreak on these monuments (Figure 1). Furthermore, the similarity of their habitat to that of other natural and man-made hypogea (show caves, subterranean churches and historical buildings), make them one of the best candidates in which to develop and apply innovative approaches to sustainable management.

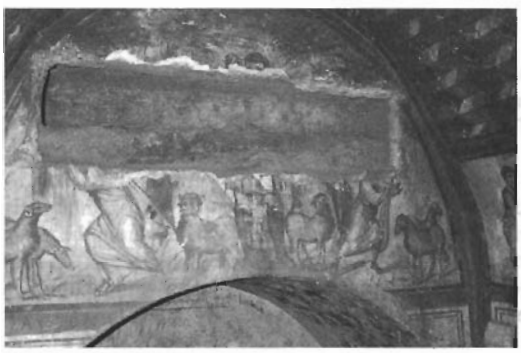

Figure 1. Frescoes in one cubiculum inside the Catacombs of St. Callixtus (Rome, Italy) with biofilms formed by different microorganisms.

In these environments the abundance of nutrients in the lithic substrata, the input of compounds from circulating air and percolating waters, and the high humidity combined with the presence of artificial illumination, provide a suitable niche for those photosynthetic microorganisms that can make use of the spectral emission of lamps (Albertano & Grilli Caiola 1989, Albertano 1993). The continual influx of visitors can, in itself, cause significant climatic changes, by providing a source of heat and of CO_2 (Andrieux 1988, Hoyos et al. 1998).

Cyanobacteria are photosynthetic microorganisms that can use CO_2 as a carbon source for growth. Many cyanobacteria, including strains found in hypogeic environments, are also diazotrophic, using N_2 as a nitrogen source.

Thanks to their peculiar ability to adapt to extremely low photon flux densities and to acclimate to a variety of spectral emissions, cyanobacteria are the major organisms responsible for biofilm formation on any rock surface (i.e. mortar, bricks, marble, frescoes, stuccoes, mosaics, etc.) exposed to light. At the same time, the availability of organic matter produced via cyanobacterial photosynthesis and N_2 fixation supports the growth of associated heterotrophic microorganisms (bacteria and fungi), the development of which contributes in a synergistic manner to the establishment of the biofilm and to the increase of the biological activity on the colonised surface (Albertano & Grilli Caiola 1990, Urzì 1993, Albertano & Urzì 1999, Dornieden et al. 2000b).

Different species of terrestrial epilithic cyanobacteria have been reported as dominant phototrophic microorganisms in natural and man-made hypogea (Hoffman 2002). Few species of eukaryotic phototrophs are able to withstand the low irradiance available in these sites, and only terrestrial species of filamentous and coccoid cyanobacteria and green algae, diatoms, and mosses have been reported (Albertano et al. 1994, 1995, Hernández-Mariné & Canals 1994, Ariño et al. 1996, 1997, Pantazidou 1997, Hernández- Mariné et al. 1999, 2001).

Microbial communities and evidences of microbial activity have been found in or on carbonate speleothems and underlying substrata in many karstic caves (Cañaveras et al. 1999). Processes of disintegration and formation of specific types of speleothems such as cave saltpetre, mineral oxide accumulations, coloration, and moonmilk deposits have been related to microbial activity. It appears that two main processes can be induced or controlled by microorganisms: destructive processes causing deterioration or surface disintegration of rocks and speleothems; and constructive processes fading or covering speleothems and rocks. Several destructive fabrics associated with microbial colonies and biofilms on cave walls and ceilings have been identified: (i) "spiky" textures on

(calcite and/or aragonite) speleothem surfaces; (ii) irregular etching on host-rock surface; (iii) "sparmicritization" and associated residual calcite and/or dolomite. Partial disintegration and covering of rock paintings also constitute destructive effects or fabrics due to microbial activity. Filamentous microbes and the (bio-) precipitation of speleothems relate constructive fabrics associated with microbial activity to the trapping and binding of detrital particles (and insect fragments). However, though some information has evidenced the relationships between microorganisms and transformation and/or erosion of calcareous surfaces, few data are available on the biological mechanisms that cause the decay of lithic substrata, especially concerning cyanobacteria.

2 THE EXPERIMENTAL APPROACH

The main objective of CATS is the evaluation of applicability of a two-phase (physical plus biotechnological) strategy to decrease and inhibit growth of biofilm forming cyanobacteria in hypogean monuments, as a tool to produce innovative non-destructive technologies to control and prevent microbial growth on rock surfaces. It has been described for other environments that the metabolic activity of cyanobacterial biofilms leads to the biotransformation and bioerosion of substrata. In archaeological hypogea, the mechanisms that cause severe damage mostly to calcareous substrata, and that are consequent to the development of phototrophic and heterotrophic microorganisms, still have to be understood. Accordingly, CATS intends to answer the following two major and essential questions in order and subsequently to develop control and preventive strategies:

- How does microbial activity alter the mineralogical,textural and geochemical features of rocks?
- What conditions limiting growth of cyanobacteria can be safety applied in Roman hypogea?

To achieve these central objectives different types of microsensors are developed to quantify biologically induced variation of gases and ions on the colonised lithic substrata. Data on the petrological and geochemical characteristics of rocks and on structure, function and diversity of biofilms are integrated with those obtained using microsensors in order to describe and model the damage of rock surfaces. This part of the project will end with the construction of a multiparametric portable device based on microsensors that will be produced as a new tool for microbial monitoring.

In parallel to the first part of the project, a new lighting system providing wavelengths poorly used by cyanobacterial photosynthesis is being tested in order to find those conditions that can drastically decrease the growth of cyanobacteria and therefore the quantity of organic matter available to the associated heterotrophic populations. Subsequently, the new lighting system will be experimentally set up *in situ* to confirm the laboratory results.

In addition to the physical approach, newly identified biomolecules of the iron metabolism and cell-to-cell signalling pathways are checked for their capability to interfere with bacterial and cyanobacterial metabolism by removing factors indispensable to microbial development. The application of these environmental biotechnologies under laboratory conditions should provide a new method to control and prevent growth of phototrophic biofilms.

2.1 *Physico-chemical deterioration processes*

Microorganisms and surface-derived organic compounds are introduced in hypogea by groundwater infiltration and by visitors (Hoyos et al. 1998, Sánchez-Moral et al. 1999). It is impossible to deny the major role of water in hypogean environments. In caves, for example, hydrochemical and environmental data processing have usually been focused on the construction of geochemical models, which have shown how parameters such as the degree of saturation of karstic waters, pH and Pco_2 control the interaction among substratum, air and water. This was especially important in evaluating the role both of microorganisms and condensation water in wall corrosion processes (Laiz et al. 1999), which are probably the most pernicious processes affecting the conservation of valuable cultural features in karstic caves. In consequence, a knowledge of the quality (chemical composition and physical properties) of infiltrational water has allowed cave researchers and managers to define: (i) the nature and rates of the dissolution/precipitation processes that take place on wall surfaces in the cave system; and (ii) the type and sources of karst groundwater pollution.

This type of analysis is therefore used in defining the contribution of water to rock decay in hypogea. In addition, analysis of both inorganic and organic pollutants in water samples, in conjunction with hydrological, geological and hydrogeological characteristics of the area studied, are used to define the sources of pollution (natural, rural, urban) (Saiz Jimenez 1994, 1995a, Saiz Jimenez et al. 1994).

2.2 *Biological deterioration processes*

Once the hypogean environments are illuminated by any natural or artificial light source, cyanobacteria become predominant causing aesthetic, physical and chemical damage (Albertano 1993) (Figure 2). At the same time, a synergistic biodeteriorative effect on stone surfaces is possibly achieved by the concomitant growth of phototrophic and heterotrophic populations,

Figure 2. Cyanobacterial biofilms visible as dark patinas on the vault and the right side of the 'Cubiculum of Ocean' inside the Catacombs of St. Callixtus (Rome, Italy). Note the heavily deteriorated frescoes in the centre as they appeared after removal of the biofilms.

as has been shown for other microbial communities in confined environments (Karpovich-Tate & Rebrikova 1991). Bacteria and fungi, in fact, should be able to use the organic matter produced by phototrophs, to release acidic organic compounds and solubilise the minerals of the substratum (Warscheid & Braams 2000). The lighting systems therefore also contribute to the development and expansion of microorganisms, thus increasing biological pollution. In general, the intensity of colonisation decreases from the entrance and the twilight zone to the interior of galleries and tunnels. The main effect is the proliferation of phototrophic microorganisms such as cyanobacteria and green algae, mosses, and in some cases plants near the light sources. Although irradiances available for photosynthetic activity can be extremely low, the growth of phototrophs is not limited, possibly because they have adapted to such low photon fluxes (sciaphilous species) or because they are able to acclimate to these conditions by adjusting photosynthesis and pigments to both the spectral composition and the intensity of prevailing light (Albertano et al. 1991a, b, Bruno & Albertano 1999). Therefore, the characterisation of light spectra utilised by cyanobacterial biofilms and studies on their capability to acclimate to various light conditions are performed in order to expressly design new lighting systems able to limit cyanobacterial growth (Bruno et al. 2001, Albertano & Bruno 2003).

Another major role is played by the strategies for the adhesion to the rock substratum that are generally based on the production and secretion at the cell surface of mucilaginous compounds. It has been shown that cyanobacterial mats develop thanks to the production of exopolymeric substances (EPS) secreted by the microorganisms (glycocalyx, sheath or envelope) (Stal 2000). The bio-polymers act in sticking the cells to the substratum, and their adhesive properties contribute to the formation and cohesion of biofilms. A polysaccharide matrix containing cell debris and significant amounts of inorganic material adsorbed from the substratum also makes up most of the cyanobacterial biofilms. To this material, airborne particles, bacteria, and spores adhere, increasingly masking the rock face, and almost certainly stimulating transformation and corrosion of the surface material (Albertano 1998). The anionic nature of exopolymers can maintain a highly hydrated, fibrous extracellular matrix, that can strongly adsorb cations and dissolved organic molecules from the minerals, and can stabilise dust particles (De Philippis & Vincenzini 1998, Albertano et al. 2000a). Calcium ions may therefore be easily subtracted from rocks of Roman hypogea and precipitated on polysaccharide sheaths of cyanobacteria in the form of calcium carbonate (Ariño et al. 1997, Schneider & Le Campion-Alsumard 1999). In addition, macronutrients such as nitrogen and phosphorus may be mobilised from the substratum and metabolised or stored in the cells (Albertano 1997). The ability of bacteria and cyanobacteria to release EPS is related to their nutritional status. In cyanobacteria, particularly, limitation in light or nutrients can increase production of polysaccharides. Since the photosynthetic activity sustains the production of new biomass into the ecosystem, cytomorphogical observations, combined with the characterisation of the photosynthetic capability of mature and developing biofilms and to the measurement of pH and released ions, are used to draw a picture of the contribution that EPS make to rock decay processes.

2.3 *Biogeochemical fluxes*

Because of their ability to identify and quantify the damage, and thus to provide an early warning for the prevention of microbial biofilms, microsensors represent a useful analytical tool for the analysis of microenvironments (Albertano & Compagnone 1999, Compagnone et al. 1999). The mechanism by which lithic surfaces decay can be understood better only when it is possible to measure chemical microprofiles within the damaging biofilm. Amperometric and potentiometric microsensors have been developed for application in aquatic environments (Revsbeck 1994,

Lassen et al. 1998), and will be now available for use on solid surfaces.

The development of electrochemical microsensors for O_2, pH, K^+, Ca^{2+}, NO_3^-, and NH_4^+ will allow application on rock surfaces in the terrestrial environment. In addition, voltammetric microelectrodes and biosensors would be developed to study Fe^{3+}, Fe^{2+} and phosphate mobilisation in biofilms. Moreover, the practical possibility of combining different types of microsensors, of miniaturising the multi-detector device and of providing instruction for its application will be tested. If successful, this will result in a new method for non-destructive measurements at valuable archaeological surfaces.

2.4 *Biological diversity*

Bacteria, and to a lesser extent fungi, are always present in the same microhabitats colonised by cyanobacteria and microalgae, and populations of heterotrophic bacteria and actinomycetes have been found in several archaeological and natural hypogea (Urzì et al. 1998, Albertano & Urzì 1999, Groth & Saiz-Jimenez 1999). The data obtained on isolated *Streptomyces* strains indicate that these microorganisms are able to grow at relatively low and stable temperatures (typically 13–15°C) and 100% relative humidity. However, an additional problem that is encountered while investigating microbial niches is that most bacteria cannot be cultured using standard microbiological methods. It has been reported that typically less than 10% of the extant microorganisms have been discovered in individual ecosystems (Ward et al. 1990). Selective enrichment cultures fail to mimic the conditions that particular microorganisms require for proliferation in their natural habitat, and there is therefore a need to perform culture-independent analysis of the composition of microbial communities. Molecular biological techniques offer new opportunities for the analysis of the structure and species composition of microbial communities (Urzì & Albertano 2001, Urzì et al. 2003a). The most recent molecular techniques like randomly amplified polymorphic DNA (RAPD), amplified rDNA restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP), restriction fragment lenght polymorphism (RFLP), and fluorescent *in situ* hybridisation (FISH) are being applied in order to provide new information on the components of microbial communities in biofilms from Roman hypogea. Some of these techniques have never before, or scarcely, been applied in the field of works of art.

2.5 *Biomolecules*

Recently, the discovery of biomolecules involved in cell-to-cell signalling has opened new possibilities for the study of microbial communities. Autoinduction has been defined as a form of intercellular communication, in which cells monitor population density *via* small autoinducer signal molecules and regulate expression of various genes in a quorum sensing manner (Davies et al. 1998, Wiggli et al. 1999). Formation of autoinducer molecules has been reported to be widely distributed among bacteria. Acylated homoserine lactone (AHL) derivatives have been identified in gram-negative bacteria, while short peptides have been detected in gram-positive bacteria and γ-butyrolactones in *Streptomyces* species. Since biofilms typically contain high concentrations of cells, cell-to-cell signals are believed to play an important role in the development of bacterial biofilms. In addition, recent findings have shown the ability to produce AHL autoinducers in bacteria isolated from prehistoric caves with wall paintings and in biofilms dominated by epilithic cyanobacteria (Bachofen 1988, Laiz et al. 1999). There is, therefore, a possibility of identifying autoinducers in biofilms growing in Roman hyopgea and, if successful, will offer a mean to influence microbial development with quorum sensing interfering substances.

Another set of biomolecules that seem to offer promising possibilities for the control of biofilms in terrestrial environment are the siderophores (Neilands 1994). These are low molecular weight, high-affinity chelators of ferric iron, synthesised and secreted by many microorganisms in response to iron deprivation. Iron is essential for the production of enzymes such as peroxidases, catalases, cytochromes, nitrate and nitrite reductase and nitrogenase, and for the synthesis of phycobiliprotein chromophores and the protein complexes of the photosynthetic pathway from PSII to ferredoxin. Iron, although abundant in most environments, is not as readily available to biological systems. Much of the accessible iron is in insoluble oxidized form, ferric hydroxide. This limitation in iron availability results in a major environmental challenge for microbes, which must double the amount of iron that they assimilate with every round of cell division (10^5–10^6 ions of Fe are required for a single bacterial cells). Nearly all aerobic and facultative anaerobic bacterial species therefore form siderophores. The siderophores spontaneously and rapidly associate with ferric iron. To surround and chelate the six-coordinate ferric iron, a molecular mass in excess of 600 Daltons is frequently required. Moreover, in the case of gram-negative bacteria and cyanobacteria this has mandated the synthesis and incorporation in the outer membrane of specific receptor structures, designed to recognise and transport the ferric siderophores. According to the literature, cyanobacteria produce mainly hydroxymate-like and catechol-type siderophores. In our case, the frequent occurrence in Roman hypogea of diazotrophic cyanobacteria offered a further motivation for the application of a control strategy based on siderophore

because of the greater potential demand for iron that such organisms exhibit. Diazotrophic cyanobacteria use the Fe-rich enzyme nitrogenase to reduce N_2 to ammonium. Since the atmosphere consists of 78% of N_2, it is not likely that nitrogen will limit their growth as is often the case for species like bacteria and other phototrophs that depend on nitrate, ammonia or organic nitrogen. Therefore, removal of available iron due to the application of antagonistic siderophores would lead at very least to a shift in the species composition of the cyanobacterial population. To identify and apply antagonistic siderophores will interfere with the iron metabolism of cyanobacteria present in Roman hypogea. Encouragement for this strategy came from the observation that fluorescent pseudomonads, commonly found in soil as effective colonizers of the rhizosphere of many crop plants, have been shown to inhibit the growth of a number of phytopathogenic fungi (Weller 1989). For example, in laboratory scale and in green house experiments *Pseudomonas marginalis* showed good antifungal activity against *Fusarium* and *Pythium*. These fluorescent *Pseudomonas* species are known to excrete, under iron stress, a family of siderophores that are yellow-green and water-soluble, and also membrane-receptor proteins that specifically recognise and take up the siderophore-iron complex (Raaska et al. 1993). These compounds were secondary metabolites whose production started after the carbon source present in the growth medium was consumed. The absorption maximum of the cell-free culture supernatants was at 400 nm (pH 7.2), which was typical for pyoverdine-like siderophores. Production of these iron (Fe^{3+})-chelating siderophores, as well as of HCN, and antibiotics, gave *Pseudomonas* spp. a competitive advantage over other microorganisms.

3 THE INVESTIGATED SITES

The Roman Catacombs of Domitilla and St. Callixtus are two Christian cemeteries, that have been carved in soft tufa rock during the first to fifth century. They are located in a volcanic area and extend along two consular roads, via Ardeatina and via Appia antica, in the southern part of Rome city (Italy). These archaeological remains are characterised by large dimensions, a network of tunnels and *cubicola* (square or rectangular chambers that were intensively used for burial), and an abundance of marble used for grave and wall decorations (mainly frescoes and stuccoes) (Figures 1–3). The Mediterranean climate of the area, with an average annual temperature of 15°C (7°C min, 24°C max) does almost not influence the inner climate of the catacombs due to their deep location (>16 m below the ground). However, since the land above is used for agriculture, fertilisers and pesticides could leach into the ground water and percolate into

Figure 3. One "arcosolium" close to the entrance of the Catacombs of St. Callixtus (Rome, Italy) during the sampling campaign on March 2001.

Figure 4. Green biofilms on the karstic geological formations of the Cave of Bats in Zuheros (Spain).

the hypogea. Domitilla and St. Callixtus are part of the 60 catacombs of Rome, each of them has about 1275 m^2 of paintings that periodically require restoration because of the growth of biofilms.

The Cave of Bats (Cueva de los Murciélagos) (Figure 4), is located at a height of 972 m a.s.l, close to the village of Zuheros (Córdoba, Southern Spain) in the northern part of Subbeticas Range. The continental Mediterranean climate of the area is characterised by a mean annual temperature of 14.9°C, ranging from −10°C in winter to more than 40°C in summer, and high rate of rainfall with maxima in November and February. The cave has been developed in Jurassic limestones of Gaena Unit. It has a maximum length of 1016 m, and a difference of 70 m high between its uppermost and lowermost part.

The cave comprises three different inter-communicating floors and is situated in the vadose zone of a karst system, the so-called senile area, in which the process of limestone dissolution/destruction is more important than precipitation/lithochemical processes. At present, the circulation of water in the cave is very scarce and it is caused by direct seepage and downward percolation of meteoric water *via* fissures and joints, that generate dripping points and discontinuous laminar flows on the cave walls. A high natural ventilation rate is provided by two openings located at both sides of the cave.

Zuheros Cave is an important archaeological site having a remarkable collection of Palaeolithic rock paintings. These paintings are attributed to two cultural Palaeolithic tendencies. One of them is characterized for carrying through drawings with red strings ant the other for carrying through black and fine strokes. Pigments studied under Raman Spectroscopy have shown two different compositions: red ones are composed mainly of hematite (iron oxide) mixed with calcite and black ones are of undetermined origin. Another interest of the cave is the amount of karstic geological formations as speleothems.

4 RESULTS AND DISCUSSION

4.1 *Deterioration processes*

Main processes that affect the archaeological sites under investigation are related to the hypogean climatic conditions and to the characteristics and composition of rock, frescoes and paintings. The host-rock of frescoes and paintings (chiefly calcite) can be altered by natural processes and/or human induced processes. Natural physico-chemical and biological processes (inorganic corrosion, water circulation, microbial activity, etc.) cause flake off of limestone walls. In addition, the combined effect of lighting and the increase in CO_2 concentration, air humidity and temperature, and the dissemination of microbes due to visitors increase the intensity and, even, the development of deterioration processes (Sanchez-Moral et al. 2003).

One of the worst problems causing deterioration of these sites stems from the fact that these hypogean environments are illuminated by artificial light sources that sustain the development of films of phototrophs (especially cyanobacteria). The preliminary results indicate that the development of microbial communities is related to growth of Actinobacteria (most frequently *Streptomyces*) favoured by the stable temperatures and high relative humidity typical of caves and hypogean environments (Groth & Saiz-Jimenez 1999). However, the presence of phototrophic biofilms has always been ascertained near to the entrances and close to the light sources. Phototrophic organisms (cyanobacteria and algae) were spread over tufa rock, marble, brick, plaster and frescoes in the two Roman catacombs and walls and speleothems of the cave in Zuheros. These cause both aesthetic and chemical damage. Three main patterns of microbial colonisation have been detected: i) green patinas (biofilms), ii) white patinas, iii) gray to black alterations. Assessment of damage carried out through microscopical analysis of adhesive tape samples and cultural analysis of biofilms showed that the abundance of phototrophic microorganisms to which heterotrophic bacteria were closely associated (Figure 5). Softening of the substrata occurred in correspondence of these biofilms, and a diffuse crystal precipitation was present at the margins of the colonised areas. White patinas were due to abundant Gram-positive rod, coccal and, especially, filamentous bacteria.

The presence of ubiquitous fungi was occasional. Grey-black alterations were present only in dark sites and caused by the development of low numbers of Gram-positive rod and coccal bacteria, and to the occasional presence of black fungi. Black spots in Zuheros cave originated from remnants of phototrophic colonisation. Streptomycetes were the most frequent filamentous actinobacteria at all sites (Table 1).

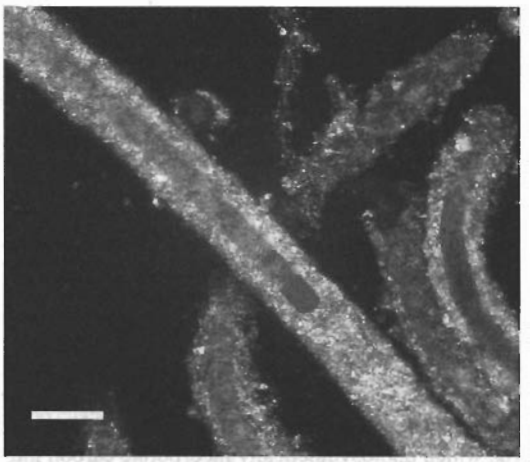

Figure 5. CLSM image of filamentous cyanobacteria forming biofilms inside Roman hypogea. Note crystals and bacteria adhering to the polysaccharides sheaths that surround trichomes. Scale bar = 10 μm.

Table 1. Distribution of isolates in hypogean environments.

Site	Domitilla	St. Callixtus	Zuheros	Total
Isolates	263	318	327	908
Gram- bacteria	36	27	31	94
Bacillus	24	19	47	90
Actinomycetes	203	272	249	724
Streptomyces	138	189	123	451

4.2 *Biogeochemical fluxes*

The presence of visitors in hypogea introduces biological pollution. The biological pollution involuntarily contributed by tourists originates from the "cloud" of spores, bacteria, skin particles, hair and lint from clothes. The entry of these organic particles, together with other "inorganic" dust particles, provide mineral salts and organic matter that facilitate the installation, colonisation and growth of bacteria, fungi and other organisms. The huge number of visitors that each year is interested to visit Roman hypogea (for instance, more than 600,000 in Rome itself), create a strong impact on climatic conditions and biological components of biofilms by increasing CO_2 and the concentration of airborne particles. An increase of CO_2, in turn, favours the development of cyanobacteria, which photosynthetically incorporate carbon into sugar molecules.

Amperometric and potentiometric microsensors have been constructed, assembled and validated to measure gases and ions within biofilms. The microsensors so far developed have been experimentally applied for oxygen, pH, calcium, potassium, and ammonium measurements (Compagnone et al. 1999, Albertano et al. 2000b, c, Calvo Quintana et al. 2002).

The respiratory and photosynthetic capability of biofilms has been assessed at increasing irradiances showing low values of photosynthesis maxima yet high efficiencies, as expected for these sciaphilous phototrophic communities. During the illumination time, the increase of pH was shown to almost parallel the increase of oxygen evolution, while calcium and potassium concentrations did not vary and showed a light-independent response. In fact, measurements performed at increasing irradiance showed that no significant variation in potassium and calcium concentration took place during the whole experiment. However, the potassium and calcium mean values measured in the collected biofilms resulted 20-50 times higher when compared with the concentrations measured in monospecific biofilms cultured in laboratory. Concerning ammonium and nitrate microelectrodes, the calibration curves of the realised microsensors showed a Nernstian response in the range 10^{-4} to 10^{-1} M with a slope of 55.0 ± 0.5 mV NH_4NO_3 for ammonium microelectrode, and a slope of -55.6 ± 0.6 mV in the range 10^{-4} to 10^{-1} M KNO_3 for nitrate microsensors.

The construction of an iron voltammetric microelectrode has been completed and preliminary application started to measure iron concentration with 5-10 μm depth resolution across the biofilms. Development of electrochemical microelectrodes to determine orthophosphate in cyanobacterial biofilms has been based on the reaction of ammonium molybdate (under acid conditions) with orthophosphate to form a heteropoly acid, the molybdophosphoric acid. This latter can be electrochemically reduced, and the resulting current is proportional to phosphate concentration.

4.3 *Biofilm structure and diversity*

Light microscopy (LM), epifluorescence, confocal laser scanning microscopy (CLSM), scanning (SEM) and transmission (TEM) electron microscopy have been used to investigate aerophytic phototrophic biofilms that developed along light gradients particularly in Roman hypogea. In lighted areas, phototrophic organisms were very abundant and cover lithic faces extensively with a patchy distribution. The general biofilm structure was porous and spatially very heterogeneous in thickness (from 80 to 550 μm) and organism composition. A general pattern in biofilm architecture could not be determined, nor a relation to the substratum composition and environmental conditions (light, humidity). Mucilaginous material contributing to the adhesion and cohesion of biofilms was always present (Albertano & Bellezza 2001). Microbial capsules and sheaths formed by exopolysaccharides (EPS) were more abundant at the upper third of most biofilms (revealed by EPS staining in 3D reconstruction and extended focus images) (Roldán et al. 2003). The cyanobacteria *Leptolyngbya* spp. and *Scytonema julianum* (Figure 6), and mosses were the main organisms responsible for the 3D structure of biofilms (Hernández-Mariné et al. 2003). Cyanobacterial filaments appeared usually erected. Among filamentous cyanobacteria, species like *S. julianum, Herpyzonema pulverulentum* and *Loriella osteophila* were characterised by the presence of carbonate precipitates on the polysaccharide sheaths that surround the trichomes, and by the differentiation of

Figure 6. ESEM image of a hydrated biofilm formed by filaments of the calcifying cyanobacterium *Scytonema julianum* tightly associated to filamentous bacteria. Scale bar = 10 μm.

heterocysts, specialised cells in which the fixation of atmospheric N_2 occurs (Bruno & Albertano 1996).

Other heterocystous, and also non-heterocystous cyanobacteria could be found growing on rock faces and in caves as the unicellular cyanobacterium *Gloeothece* and the heterocystous cyanobacterium *Nostoc* (Griffiths et al. 1987, Flynn & Gallon 1990, Bellezza & Albertano 2003). Both fixed N_2 but the former taxon could clearly be distinguished from the latter by the diurnal pattern of nitrogenase activity. *Gloeothece* fixed N_2 at night, during the period of darkness, whilst *Nostoc* fixed N_2 during the day. For cyanobacteria growing on stone surfaces, whether natural or man-made, the N-deficient nature of the substrate would favour the establishment of diazotrophic (N_2-fixing) species. This is not always the case, however. It is possible, for example, that the non-diazotrophic cyanobacteria found in these environments are very efficient at scavenging traces of fixed N_2, such as nitrate and ammonia, either from the atmosphere or from water that percolates to the site of growth. Alternatively, heterotrophic bacteria that occur as components of the complex microbial community of which the cyanobacteria are only one component, may fix N_2 (Paerl 1992).

The microbiological characterization of biofilms from Roman catacombs and Zuheros cave allowed to isolate and identify most of the representative heterotrophic microorganisms present in dark sites and/or associated to algal colonization in illuminated areas. The development and application of Single Strand Conformational Polymorphism (SSCP) and Fluorescent In Situ Hybridization (FISH) molecular techniques to study the diversity of the microbial community is providing a rapid and useful method for an *in situ* direct identification of microorganisms at genus level (Urzì et al. 2002, 2003b).

4.4 *Biomolecules*

Quorum-sensing molecules functioning as a chemical signal between different partners in the biofilms, and allowing coordinated growth and development of the entire system were identified. Disruption of cell-to-cell signals might be a valuable means of inhibiting the formation and development of biofilms. Using both chemical and function-based assays, it has been shown that all of the cultures of cyanobacteria isolated from the catacombs of Rome produce quorum-sensing molecules of the N-acyl homoserine lactone type. At this stage it is not clear whether it is the cyanobacteria themselves or contaminating microorganisms or both that are responsible for N-acyl homoserine lactone production.

Siderophores, low molecular weight high-affinity ferric iron chelators, have been produced by non-pathogenic bacteria (*Staphylococcus carnosus* and *xylosus*) under low-iron conditions in deferrated growth media. The siderophore preparations inhibited growth of Gram-negative catacomb isolates.

The alternative strategy based on biomolecules could lead to the control and/or prevention of phototrophic biofilm development on rock surfaces. Obviously, in order to succeed in such a biotechnological strategy it is necessary to know how the different species are involved in the decay processes and which are the most damaging, and CATS will provide these data. However, there are additional problems that will not be addressed in this proposal, related to the fate of these biomolecules *in situ*. Nevertheless, in combination with other classical or new conservation techniques the "antagonistic siderophores" and the "interfering quorum sensing" methods would offer a good chance of eliminating damaging biofilms and represent a totally new approach capable of further development. The use of a biological anti-cyanobacterial strategy has never been attempted before, and the possibility of applying either siderophores or biomolecules that interfere with biofilm formation would be extremely promising.

5 CONCLUSION

Protection and management of the artistic legacy found in hypogean monuments has been addressed by a multidisciplinary scientific study. This approach includes geological, hydrochemical, microclimatic, environmental, and microbiological studies. The combination of all these is being used to construct a representation of the complexity of the hypogean environment and of the biological-mediated decay processes occurring inside it.

Notwithstanding, the elimination of microbial colonisation from decorated walls in Roman hypogea seems to be a complex and difficult task. The typical methods used to restore speleothems (for example the cleaning with butyl alcohol or bleaching solutions) would provoke adverse or irreversible effects on rock-paintings and cannot therefore be taken into account. On the other hand, pesticides that act against bacteria and cyanobacteria have been tested *in situ* and laboratory conditions, but the periodic need to repeat these treatments, the unpredictable shift that can be caused in the microbial populations during re-colonisation, and the possible chemical interactions with decorated substrata have discouraged this approach. Moreover, chemical compounds that are toxic for microorganisms cannot be safely employed because of the serious health risks connected with their use in confined environments such as hypogea, which have limited air circulation and a massive influx of visitors.

On this basis, the development of non-destructive and safety methods for control and prevention of cyanobacterial biofilms has been started. The combination of a physical and biotechnological two-phase

strategy offers the doubled chance of providing tools for both control and prevention of the growth of phototrophic organisms. Testing the individual citizen's response and expectation, both towards perceived problems in Roman hypogea and to innovative solutions to these problems, will be the last step of the project and essential for the initiation of a policy of integrated intervention between conservation managers and users.

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